Research Article

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14-3-3η is a novel growth-promoting and angiogenic factor in hepatocellular carcinoma

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Background & Aims: Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. The continued search for novel therapeutic strategies for HCC is urgently required. In this study, we aimed to investigate the functions and clinical significance of 14-3-3η protein in HCC.

Methods: Expressions of genes and proteins were determined by quantitative reverse transcription polymerase chain reaction, Western blot, and immunohistochemistry. Their functions were assessed by endothelial cell recruitment, tube formation, wound healing, flow cytometry, immunostaining, immunoprecipitation, and xenograft assay. A tissue microarray followed by univariate and multivariate analyses was performed to indicate the clinical significance.

Results: In HCC specimens, overexpression of 14-3-3η was observed not only in tumors but also in intratumoral vessels. In HCC and vascular endothelial cells, 14-3-3η stimulated proliferation and angiogenesis, but attenuated the functions of sorafenib. Briefly, 14-3-3η facilitated the phosphorylation of extracellular signal-regulated kinase1/2 (ERK1/2). Then, by binding to the phosphorylated-ERK1/2 (p-ERK1/2), formed a functional positive feed-back loop. A xenograft model showed that, blockage of either 14-3-3η or ERK1/2 inhibited the tumor growth. Finally, tissue microarray analyses showed that overexpression of 14-3-3η, either in tumors or intratumoral vessels, contributed to the poor survival.

Conclusions: The 14-3-3η-ERK1/2 feedback loop played a characteristic growth-promoting role in HCC, not only in tumors but also in intratumoral vessels. Further, 14-3-3η could be a potential therapeutic target for HCC and a biomarker for predicting sorafenib treatment response.

Lay summary: Here we found that, 14-3-3η protein exhibited a characteristic growth-promoting effect in both tumor and intratumoral vessels of hepatocellular carcinoma by interacting with ERK1/2 signaling.

Keywords: Hepatocellular carcinoma; 14-3-3η; Extracellular signal-regulated kinase1/2; Tumor growth; Angiogenesis.

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Abbreviations: Cxcl1, C-X-C motif-ligand 1; ECs, endothelial cells; EMT, epithelial to mesenchymal transition; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FRA-1, fos related antigen 1; NK-κB, nuclear factor kappa B; PCNA, proliferating cell nuclear antigen; TGF-β, transforming growth factor beta; TUNEL, transferase-mediated deoxyuridine triphosphate-biotin nick end labeling; VEGF, vascular endothelial growth factor; Zeb-1, zinc finger E-box-binding protein-1.

Introduction

Hepatocellular carcinoma is one of the most common solid tumors and the second leading cause of cancer-related mortality worldwide [1,2]. Current standard curative practices for treatment of HCC are the liver resection or liver transplantation, however, the long-term outcome after these therapies is less than satisfactory because of the high post-surgical recurrence [3]. Consequently, the continued search for novel therapeutic strategies for HCC is urgently needed.

The 14-3-3 proteins are a family of approximate 28 to 33 kDa acidic polypeptides; there are seven mammalian isoforms of the 14-3-3 protein (α/β, γ, σ, ε, ζ, η, and θ/τ), which regulate multiple cellular functions via interactions with intracellular proteins by phosphoserine and phosphothreonine bindings [4,5]. Studies indicate that 14-3-3 proteins can regulate cancer cell proliferation, survival, migration/invasion, and function as potential therapeutic targets [6,7]. Up to date, five members of 14-3-3 family proteins (α/β, γ, σ, ε, and ζ) have been identified to be involved in the HCC progression, including tumor growth, metastasis, and resistance to sorafenib [8–13], however, the functions and clinical significances of 14-3-3θ/τ and η in HCC are still largely uninvestigated.

Cancer

Here we identified that overexpression of 14-3-3η was observed both in HCC tumors and intratumoral vessels, and that, overexpression of 14-3-3η prognosticated poor overall/recurrence-free survival. In the context of the molecular mechanisms involved, 14-3-3η and phosphorylated extracellular signal-regulated kinase (p-ERK)1/2 formed a functional positive feed-back loop, which enhanced cellular proliferative and angiogenic abilities, but attenuated the functions of sorafenib. Collectively, our findings have provided the underlying mechanisms of 14-3-3η in the growth and angiogenesis of HCC, and implicated 14-3-3η as a potential prognostic biomarker and therapeutic target for HCC.

Materials and methods

Patients and tissue microarray

This study was approved by Medical Ethics Committee of Nanjing Medical University, and the participants' written informed consents were obtained from each patient for the study of tissue excised from surgical specimens. A cohort of 256 Chinese HCC patients was enrolled in this study, the clinic-pathologic data was listed in Supplementary Table 1. To validate the clinical significance of 14-3-3η in HCC, we performed a tissue microarray constructed by Shanghai Zhuoli Biotechnology Co., Ltd (Zhuoli Biotechnology Co, Shanghai, China). In each case, 1–2 μm thick sections from paraffin tissue blocks were cut, dewaxed, pretreated and transferred to glass slides used with an adhesive tape transfer system, in order to carry out ultraviolet cross linkage. All reactions were performed on an automated staining device. The quantitation of immunostaining for 14-3-3η was completed by two independent researchers who were blinded regarding patient details. The immunostaining score of 14-3-3η in tumor parenchyma was semi-quantified by Quick-score (Q-score) based on intensity and heterogeneity [13]. The positive rates were scored as 0 point (0%), 1 point (1–25%), 2 points (26–50%), 3 points (51–75%), and 4 points (76–100%). The score of the staining intensity was presented as 0 point (none), 1 point (low), 2 points (medium), and 3 points (high). The Q-score was the sum of heterogeneity and intensity. The expression was defined as positive/high when the combination scores were $\geqslant$2/4. The immunostaining in intratumoral vessels was merely scored intensity (none, low, medium, and high to 0, 1, 2, 3) and the expression was defined low when the score $\leqslant$1.

Cell culture and transfection

HepG2, HuH7, and human umbilical vein endothelial cell line (HUVECs), were obtained from Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The MHCC97H cells were obtained from the Liver Cancer Institute, Zhongshan Hospital, Fudan University (Shanghai, China). These cells were identified by China Center for Type Culture Collection (Wuhan, China). Cells were maintained in a 37 °C humidified incubator with 5% CO_2. HepG2, HuH7, and MHCC97H cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies/Gibco, Grand Island, NY), while HUVECs were cultured in extracellular matrix (ECM) medium (Invitrogen, Carlsbad, USA). The mediums were supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 100 μg/ml heparin, and 30 μg/ml ECs growth supplement (for HUVECs, Sigma-Aldrich, MO, USA). Phenol red was added into the medium to reflect the pH. A mycoplasma stain assay kit (Beyotime Co. Ltd, Haimeng, China) was used for mycoplasma testing. For cell transfection, the pcDNA-3.1-14-3-3η-FLAG plasmid that overexpressed both 14-3-3η and FLAG was created by inserting the coding sequences of 14-3-3η (YWHAH, 741 bp) into pcDNA3.1 plasmid, followed by adding a FLAG-tag at its N-terminal (Generay Biotech Co. Ltd, Shanghai, China). The commercial specific 14-3-3η small interfering (si)RNA was purchased from Santa Cruz Biotechnology (http://datasheets.scbt.com/sc-43581.pdf). Cells were transiently transfected using the Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's protocol. Briefly, cells were seeded in 6-well plates at a density of 1×10^5 per well. After 24 h, these cells were transfected with 5 ng/ml vector-Con or 14-3-3η-FLAG, or 20 nM si-Con or si-14-3-3η for 12 h. After transfection, such cells were cultured in fresh medium supplemented with 10% FBS for another 24 h before being used for other experiments.

Animals and xenografts

This study was approved by Nanjing Medical University Institutional Animal Care and Use Committee, and animals were treated humanely and with regard for alleviation of suffering. Briefly, the BALB/c nude mice were obtained from SLRC laboratory animal center (Shanghai, China), and kept in a specific pathogen-free and temperature-controlled environment (20–22 °C) with a 12 h light dark cycle and with free access to drinking water and chow. For xenograft study, 2×10^6 cells in 100 μl matrigel were injected subcutaneously into the right armpit of the mice (5 mice per group) for 3 weeks. To determine the effects of 14-3-3η or ERK1/2 on the *in vivo* growth of HCC, we performed the intratumoral injection assay. Briefly, 100 μl of siRNA (si-Con or si-14-3-3η, 100 nM) or 100 μl of ERK1/2 inhibitor, U0126 (20 μM, Beyotime) were intratumoral injections every 3 days. Tumors were measured every 3 days and their volumes were calculated using the formula: $V = \frac{1}{2}(\text{width}^2 \times \text{length})$. After 21 days, the mice were sacrificed, and tumor tissues were removed for further investigation.

Please see the Supplementary materials and methods section for additional procedures.

Results

Identification of 14-3-3η as a characteristic cancer-promoting factor in HCC

We initially evaluated the expression of 14-3-3η in HCC patients. As shown in Fig. 1A, compared with adjacent non-tumor liver tissues, a considerable elevation of *14-3-3η* mRNA was observed in HCC tissues (Fig. 1A). Moreover, overexpression of 14-3-3η protein was also demonstrated in HCC tissues by immunohistochemistry (IHC) (Fig. 1B; Supplementary Fig. 1). Interestingly, in addition to the well-defined location in tumor cells, 14-3-3η showed a continuously capillary endothelial staining, which exhibited a different characteristics compared to the other six family members (Supplementary Fig. 2). As HCC is a highly vascularized tumor [14], we hypothesized that the higher expression of 14-3-3η might be associated with more aggressive types of tumors. To confirm this hypothesis, we then divided these HCC specimens into two groups ("14-3-3η low" *vs.* "14-3-3η high", Supplementary Fig. 3). As shown in Fig. 1C, the transverse diameters of tumors in 14-3-3η high group were significantly larger than those in 14-3-3η low group. Moreover, compared with well differentiated HCC tissues, overexpression of 14-3-3η was observed in poorly differentiated HCC tissues (Fig. 1D, E; Supplementary Fig. 4). A stronger staining for 14-3-3η was also observed with increasing TNM Classification of Malignant Tumors stage (Fig. 1F, G, and Supplementary Fig. 5). Collectively, these results suggested that 14-3-3η had a characteristic role in leading HCC progression at least in part through promoting the growth and neovascularization both in carcinoma and vascular ECs.

Effects of 14-3-3η on the proliferative, anti-apoptotic, and angiogenic abilities in HCC cells

We analyzed the expression of 14-3-3η in a panel of well-established human HCC cell lines [15]. As shown in Supplementary Fig. 6, the expressions of 14-3-3η were increased in six HCC cell lines compared to the non-transformed hepatic cell line, L02. Interestingly, in HepG2 cells (relative lower expression of 14-3-3η), forced expression of 14-3-3η shortened the doubling time and promoted the cell cycle transition (Fig. 2A and B). Moreover, these cells exhibited increased expressions of proliferating